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Interactions of a tetravalent branched peptide from VP3 capsid protein of hepatitis A virus with monolayers as biomembrane models

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The interactions between a synthetic multiple antigenic peptide containing four units of a peptide corresponding to the sequence (110–121) of VP3 protein of the hepatitis A virus, termed MAP_4–VP3(110–121), and phospholipids as the main components of biological membranes have been studied in detail. Surface activity of the multiple antigenic peptide was determined as a function of its bulk concentration in an aqueous solution. Saturation was reached at 0.33 μM concentration. The ability of the peptide to insert into lipid monolayers of dipalmitoyl phosphatidylcholine, dipalmitoyl phosphatidylglycerol and stearyl amine was determined. The peptide interacts preferably with the positive phospholipid according to its negative charge.

(Keywords: hepatitis A; multiple antigenic peptide; phospholipids; monolayers)

INTRODUCTION

The hepatitis A virus (HAV) has a world-wide distribution. Infection rates are highest in circumstances of poor sanitation and crowded living conditions. The most common mode of HAV transmission is through close personal contact, usually by the oral–faecal route.

There is no specific treatment for hepatitis A other than supportive care. Therefore the best therapy is prevention, by stopping transmission of the virus and/or rendering susceptible individuals resistant to infection by active immunisation[1].

The use of new strategies based on synthetic peptides which can elicit an efficient immune response offers the advantage of high purity, defined structure and safety. Multiple antigen peptide systems (MAPs) have provided immunological reagents for analysis and induction of the cellular and humoral immune response to a wide range of infectious diseases[2]. In contrast to the carrier-dependent immunogenicity of linear peptides, MAPs are immunogenic in the absence of a protein carrier. MAP systems are branched compounds based on a core of lysine residues containing both α- and ε-amide linkages between themselves. By sequential propagation with these amino groups of lysine a branched scaffolding can be constructed onto which antigenic peptide sequences can be synthesised.

The specific interactions between bioactive peptides and membrane phospholipids control the processes of antigen presentation and recognition in immunogens. Monolayers can provide a simple and good model to study these interactions[3–6].

In the present work, a detailed study of the interactions between a tetravalent branched peptide from the VP3 capsid protein of the hepatitis A virus and neutral, positive and negative phospholipids, as the main components of biological membranes, is reported.

EXPERIMENTAL

Chemicals

N-α-fluorenylmethoxycarbonyl amino acids and *p*-benzyloxybenzyl alcohol resin (Wang resin) were obtained from Novabiochem, UK. Dimethylformamide (DMF) and piperidine/DMF (20%) were from Milligen. Washing solvents such as isopropyl alcohol, acetic acid and diethyl ether were obtained from Merck (pa). trifluoroacetic acid

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(TFA) was supplied by LKB (Ultrosyn Chemicals) and scavengers such as anisole, *m*-cresol, thioanisole and ethane dithiol were from Fluka and Novabiochem, respectively.

Amino acid analyses were carried out in a Pico-Tag system (Waters).

High-performance liquid chromatography (HPLC) was performed in a Perkin–Elmer series 250 LC pump connected to a LC-235 diode array detector and an LCI-100 integrator. Analytical HPLC was performed on a 250 mm×4 mm Spherisorb ODS-2 (10 μm) column eluted with several mixtures of acetonitrile (0.1% TFA)/water (0.01% TFA) with a flow rate of 1 ml min^{-1}.

Dipalmitoyl phosphatidylcholine (DPCC), dipalmitoyl phosphatidylglycerol (DPPG) and stearyl amine (SA) were from Sigma, and used without further purification. Phosphate buffer saline (PBS) solution at pH 7.4, conductivity 15.4 mS cm^{-1}, was prepared.

Synthesis of multiple antigenic peptide MAP_4–VP3(110–121)

MAP_4–VP3(110–121) (*Figure 1*) was synthesised manually, as described previously[7]. Briefly, 2 mmol of Fmoc–Ala, previously activated as its symmetrical anhydride or by use of pyBop reagent, was sterified onto 2 g of Wang resin (0.8 meq g^{-1}). This reaction was carried out to a low extent, in order to achieve anchoring values of about 0.1 meq Fmoc–Ala per gram of resin. Unreacted groups were acetylated by reaction with Ac_2O (2 eq) in DMF in the presence of 1 eq of 4-(dimethylamino)piridine (DMAP) for 30 min. The tetravalent lysine core was obtained by sequential coupling of 0.3, 0.6 and 1.2 mmol amounts of Fmoc–Lys(Fmoc), which were incorporated through diisopropylcarbodiimide/hydroxybenzotriazole. The stepwise addition of each residue was assessed by the ninhydrin colour test. The peptide was cleaved from the resin by treatment with TFA:ethanedithiol:H_2O, 75:20:5 (2.5 h, room temperature), the solvent was removed *in vacuo* and the crude peptide was precipitated with ether. The sample was sonicated and centrifuged and the supernatant decanted off, repeating this last step several times. The crude peptide was dialysed in 8 M urea and then twice in 0.1 M acetic acid for 5–6 h to remove the urea. Finally, the peptide was purified by semi-preparative HPLC.

The final peptide was characterised by analytical HPLC, amino acid analysis and electrospray mass spectrometry.

Monolayer studies

Measurements at constant surface area. A mini Teflon trough, cylindrical in shape and with capacity of 70 ml, was used. Throughout this study, the aqueous subphase was PBS (pH 7.4, conductivity 15.4 mS cm^{-1}, 313.35 mosm kg^{-1}).

Measurements of surface activity. The surface activity of the peptide was studied by using the minicuvette described above. To measure the equilibrium spreading pressures, increasing volumes of concentrated solutions of peptide were injected beneath the aqueous surface (PBS), and pressure increases after 60 min were recorded.

Penetration of peptide into monolayers. Peptide penetration into the monolayers was determined by injecting the peptide into the subphase under a previously formed monolayer of DPPC, DPPG and SA spread at different initial surface pressures (5, 10 or 20 mN m^{-1}). Pressure increase was measured for 60 min.

Compression isotherms. Compression isotherms were recorded on a Langmuir film balance equipped with a

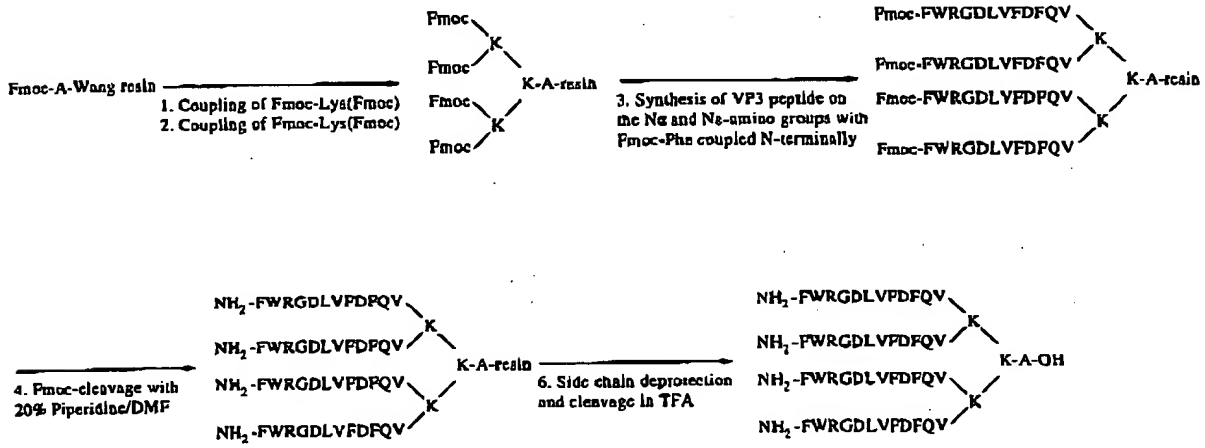

Figure 1 Synthesis strategy of MAP_4–VP3(110–121)

Wilhelmy platinum plate as described by Verger and De Haas[8]. The output of the pressure pickup (Sartorius microbalance) was calibrated by recording the well-known isotherm of stearic acid. The Teflon trough (surface area 495 cm^2, volume 330 ml) and platinum plate were cleaned regularly with hot chromic acid.

Films composed of peptide or lipids (dissolved in chloroform) were spread on aqueous surfaces by using a Hamilton microsyringe, and at least 10 min was allowed for solvent evaporation. Monolayers were compressed at a constant rate of 4.2 cm min^{-1}. Changes in the compression rate did not alter the shape of the isotherms. All the isotherms were run at least three times in the direction of increasing pressure with freshly prepared films. The accuracy of the system under the conditions for which the bulk of the reported measurements were made was 0.5 mN m^{-1} for surface pressure. All the measurements were made at 21±1°C.

RESULTS

Synthesis

MAP_4–VP3(110–121) was successfully synthesised following Fmoc strategy with a small variation of standard protocols. Crude peptide was purified by semi-preparative HPLC and gave consistent amino acid analysis and electrospray mass spectra.

Peptide characterisation

Surface activity. The peptide containing four copies of the 110–121 fragment of VP3 protein was selected according to the hydrophilicity profile of the whole VP3 protein, generated by applying the method of Hopp and Woods[9], owing to the presence of the only RGD sequence existing in the whole protein primary structure of the HAV capsid.

The surface activity of the peptide was determined by injecting different volumes of a standard solution (1 mg ml^{-1}) beneath the surface of phosphate-buffered saline and recording the surface pressures achieved. The peptide showed a gradual incorporation at low concentration. When higher concentrations of MAP were injected the incorporation of peptide into the surface was very fast. It took only 5 min to reach almost 90% of the final surface pressure achieved. The higher the peptide concentration in the subphase, the faster the incorporation of the peptide to the interface. Nevertheless, experiments were carried out by leaving 60 min to ensure that the system had reached the equilibrium. Results are given in *Figure 2* and *Figure 3*.

From these values the spreading pressure can be considered to be 15.18 mN m^{-1}, and corresponds to an initial concentration of peptide in the bulk phase of 0.33 μM. The surface excess calculated was 5.6×10^{-10} mol cm^{-2}.

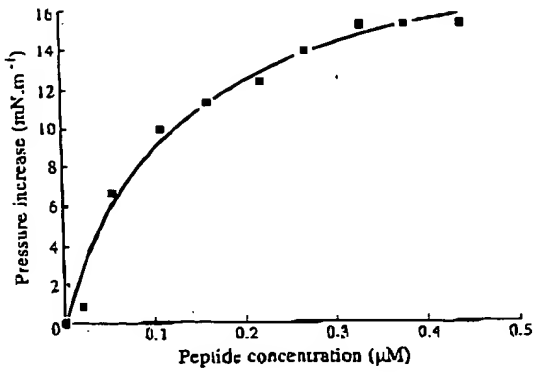

Figure 2 Surface pressure increases of MAP_4–VP3(110–121) represented as a function of the peptide concentration in the subphase

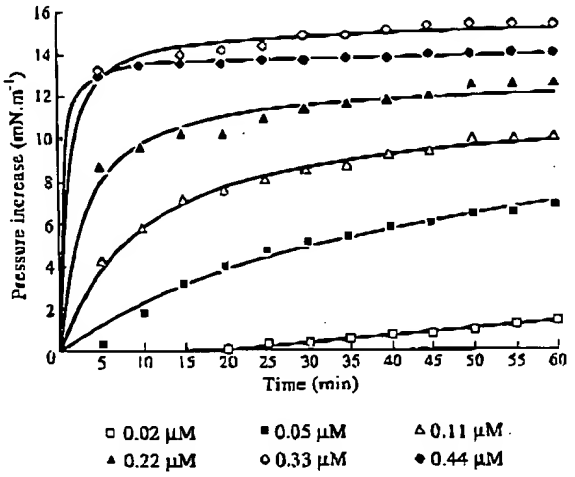

Figure 3 Monolayer surface pressure increase for several initial peptide concentrations in the subphase induced by MAP_4–VP3(110–121)

Comparing the surface activity of the synthetic tetrameric branched peptide with the surface activity previously obtained for the corresponding linear peptide[10], similar results were obtained. However, 10 times less MAP_4–VP3(110–121) was required to reach the maximum surface pressure. This result could be in agreement with the steric and globular differences between linear and branched peptides. In fact, when the surface properties of the tetrameric MAP corresponding to the sequence (11–25) of the HAV-VP1 protein was studied, the incorporation of peptide to the surface was also dependent on concentration until saturation at a point corresponding to a bulk concentration close to that obtained for MAP_4–VP3(110–121)[11].

In order to obtain more information about the surface activity of this peptide, we have assumed that the increase in surface pressure, Π, is a function of the

amount of peptide accumulated at the surface and that increases in Π can be either a linear function of time, t [equation (1)], or follow a Lineweaver–Burk curve [equation (2)].

$$\Pi = a + bt \quad (1)$$

where a and b are constants, and

$$1/\Pi = K/\Pi_m t + 1/\Pi_m \quad (2)$$

where Π is measured in $nM\,m^{-1}$, t is in min, and the calculated values of Π_m and K represent the maximum pressures achieved at the time to reach saturation and the time to reach half maximum saturation ($t_{1/2}$), respectively. The results are given in *Table 1*.

Monolayer binding properties. The ability of MAP_4–VP3(110–121) to insert into phospholipid monolayers was assessed by injecting the peptide beneath a monolayer spread at different initial surface pressures and monitoring the consequent changes in the surface pressure, while holding the surface area constant.

The concentration of peptide in the subphase was 0.22 μM. This value was selected according to the surface activity values described above and corresponds to a point slightly lower than the spreading pressure of the peptide.

The experiment was carried out for monolayers composed of DPPC, DPPG and SA, spread at initial surface pressures of 5, 10 and $20\,mN\,m^{-1}$ (*Figure 4*). As usual, the higher the surface pressure, the lower the pressure increase promoted by the peptide insertion into the monolayer. Mathematical treatment of the results obtained is given in *Table 2*.

The differences observed in penetration are dependent on the monolayer composition. According to the net negative charge of MAP_4–VP3(110–121), the electrostatic interactions between the peptide and the positively charged phospholipid influence the incorporation of the peptide molecules into monolayers. As shown in *Figure 5*, the peptide interacts preferently with stearyl amine and a lower interaction with neutral and negatively charged monolayers is observed.

Compression isotherms. In another set of experiments, the compression isotherms of the peptide were determined.

Table 1 Parameters of equations (1) and (2)

c (μM)	a ($mN\,m^{-1}$)	b	r^2
0.02	20.02±0.66	0.02±0.001	0.981
c (μM)	Π_m ($mN\,m^{-1}$)	K (min)	r^2
0.05	11.89±1.43	44.43±10.09	0.976
0.11	11.23±0.18	9.47±0.61	0.996
0.22	12.52±0.24	2.96±0.45	0.984
0.33	15.21±0.14	0.98±0.16	0.994
0.44	13.85±0.04	0.35±0.04	0.999

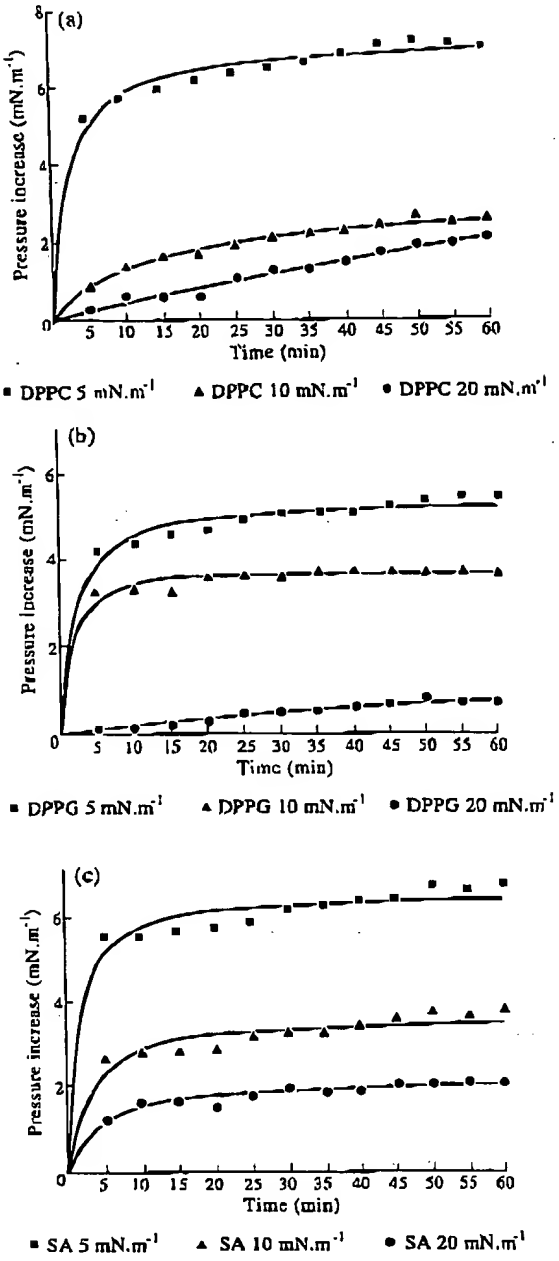

Figure 4 Pressure increases recorded after injection of MAP_4–VP3(110–121) solution under (a) DPPC, (b) DPPG and (c) SA monolayers spread at 5, 10 and $20\,mN\,m^{-1}$ initial surface pressure

The ability of the peptide to form stable monolayers was checked as follows. The peptide (1.5 to 23 nmol) was spread on the subphase and compression started after 10 min of spreading. In *Figure 6* the compression isotherm corresponding to a MAP concentration of 7.4×10^{-2} M spread on PBS is given. One can appreciate a common change from liquid expanded to liquid condensed states at $5\,mN\,m^{-1}$. The solid state was not observed and collapse pressure was achieved at $38\,mN\,m^{-1}$. The

Table 2 Parameters of Equation 2

Π_0 (mN m^{-1})	Π_m (mN m^{-1})	K (min)	r^2
DPPG			
5	5.57±0.10	2.24±0.40	0.982
10	3.88±0.05	1.40±0.24	0.990
20	5.83±0.86	335.47±513.7	0.930
DPPC			
5	6.99±0.16	2.02±0.47	0.973
10	3.05±0.12	14.4±1.86	0.983
20	11.78±6.65	286.22±187.20	0.979
SA			
5	6.56±0.13	1.42±0.38	0.976
10	3.70±0.13	2.97±0.84	0.944
20	2.18±0.07	4.40±0.88	0.965

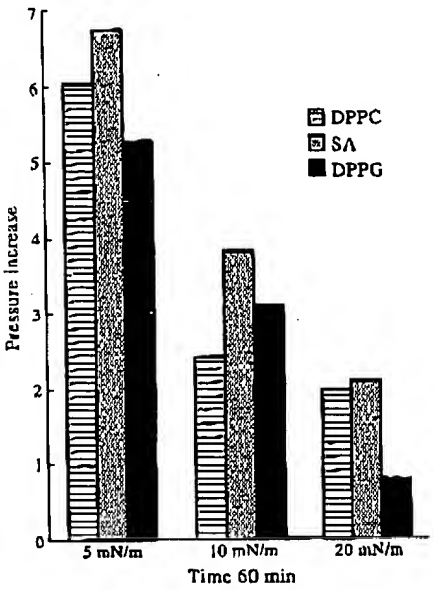

Figure 5 Comparison of maximum pressure increases recorded after injection of MAP_4–VP3(110–121) solution under (a) DPPC, (b) DPPG and (c) SA monolayers spread at 5, 10 and 20 mN m^{-1} initial surface pressure

area per molecule value obtained at 10 mN m^{-1} was 0.7 nm^2 molecule^{-1}. According to data given in the literature[12], this area can be associated with a β-sheet structure.

The physicochemical data obtained for the studied multiple antigenic peptides confirm the ability of these macromolecular structures to interact with phospholipids as the main components of biological membranes to a greater extent than linear peptides, thus indicating that branched peptides could provide a good alternative for the development of a better interaction between immunogenic peptides and antigen presenting cells.

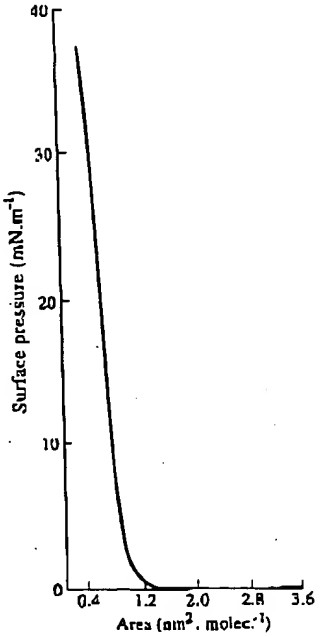

Figure 6 Compression isotherm of MAP_4–VP3(110–121)

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